Investigational New Drugs 1, 103-115 (1983)
© 1983, Martinus Nijhoff Publishers. Printed in the Netherlands

# Inhibitory activity of diarylamidine derivatives on murine leukemia L1210 cell growth

Jan Balzarini,[1] Erik de Clercq[1] and Otto Dann[2]

[1] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium*

[2] *Institut für Pharmazie und Lebensmittelchemie, Friedrich-Alexander Universität, Erlangen D-8520, F.R.G.*

Key words: diarylamidines, diarylimidazolines, murine leukemia L1210, DNA synthesis

Ref. # 15

## Summary

A series of 96 diarylamidine (and diarylimidazoline) derivatives were evaluated for their inhibitory effects on the growth and DNA synthesis of murine leukemia L1210 cells. The amidino- and imidazolino-substituted aryl moieties of the compounds consisted of phenyl, indole, indene, benzofuran, benzo[b]thiophene or benzimidazole. Several of these compounds were found to inhibit L1210 cell proliferation with an $ID_{50}$ (50% inhibitory dose) of 1 μg/ml or lower. Structure-function analysis revealed that the antitumor cell activity of the diarylamidines depended on the planarity of the molecule, the presence of amidino- (or, preferably, imidazolino-) groups on both aryl moieties, the nature of the bridge connecting the two aryl moieties (preferably no bridge at all, phenoxy or ethene) and, finally, the nature of the aryl moieties (preferably, benzofuran or benzo[b]thiophene). Hence, compound *20* (6-(2-imidazolin-2-yl)-2-[4-(2-imidazolin-2-yl)phenyl] benzo[b]thiophene) emerged as the most potent inhibitor of L1210 cell growth ($ID_{50}$: 0.21 μg/ml). Its inhibitory potency was similar to that of the well-known trypanocidal drug ethidium bromide (compound *98*). For all diarylamidine derivatives taken together, some correlation ($r = 0.612$) was noted between the log $ID_{50}$ for L1210 cell proliferation and the log $ID_{50}$ for L1210 cell DNA synthesis (as monitored by [*methyl*-$^3$H]dThd incorporation). These findings suggest that the inhibitory effects of the diarylamidines on L1210 cell proliferation may at least partially reside in an inhibition of DNA synthesis. Compound *41* (2,2′-vinylenedi-1-benzofuran-5-carboxamidine), that exhibited a potent antitumor activity *in vitro* ($ID_{50}$: 1.5 μg/ml), was further evaluated for its antitumor efficacy *in vivo* and found to increase the median survival time of L1210 cell-inoculated $BDF_1$ mice up to 204%, if administered at a dose of 200 mg/kg.

## Introduction

Aromatic diamidines such as stilbamidine and pentamidine inhibit the growth of protozoa, bacteria, fungi and tumor cells, generally at concentrations well below those found to be toxic for the host (1). Another diamidine derivative, DAPI (4′,6-diamidino-2-phenyl-indole), has been shown to form fluorescent complexes with double-stranded DNA (2, 3) and can be used for the fluorescent staining of bacteria, spermatozoa, mycoplasmata (4) and chromosomes (5). DAPI and its congeners strongly interact with A-T regions of DNA (6–8), thereby suppressing the DNA-directed RNA and DNA polymerases, and this inhibitory activity may obviously contribute to the growth-inhibitory effects that have been observed with diarylamidines in various cell systems. Several diarylamidines are potent inhibitors of reverse transcriptase (RNA-directed DNA polymerase) (9) and arginine-specific proteases (trypsin, thrombin, kallikrein) (10), and while there is no direct correlation between

*Address for reprints*: Prof. Dr. Erik de Clercq, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.

BEST AVAILABLE COPY

anti-DNA polymerase activity and anti-protease activity (9), both activities depend on rather specific structural requirements.

We have now evaluated a large series of diarylamidine (and diarylimidazoline) derivatives for their inhibitory effects on the growth of murine leukemia L1210 cells. Because of their strong affinity for DNA, the compounds were also evaluated for their inhibitory effects on cellular DNA synthesis (monitored by [*methyl*-$^3$H]dThd incorporation). These studies were aimed (i) at delineating the structural features that determine the cell growth-inhibiting activity of diarylamidine derivatives, (ii) at gaining further insight in their mechanism of cytotoxic action, and, hopefully, (iii) at detecting some compounds with potential anticancer activity.

Based on the structure-function analysis one should be able to establish the guidelines for the synthesis of new and more potent inhibitors of tumor cell proliferation.

## Materials and methods

*Cells.* Murine leukemia L1210 cells were grown in 75 cm$^2$ tissue culture flasks (Falcon 3024F; Becton Dickinson France S.A., Grenoble, France) in Eagle's minimal essential medium, supplemented with 10% (v/v) inactivated fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland), 0.075% (w/v) $NaHCO_3$ (Flow Laboratories) and 25 units/ml of nystatine (S.A. Labaz N.V., Brussels, Belgium). The L1210 cell line was found to be *Mycoplasma*-free.

*Chemicals.* The diarylamidine derivatives listed in Tables 1 through 6 were synthesized as described previously. The references for the synthesis of the compounds are as follows: *1–4, 9, 26* (ref. 11); *11, 19, 21–23, 75–77, 83, 88, 95* (ref. 12); *14–17, 24, 25* (ref. 13); *30–34, 49, 65* (ref. 14); *39–41, 44–46, 52, 55–57, 96* (ref. 15); *10, 12, 13, 42, 43, 47, 48, 50, 51, 78–82, 84–87* (ref. 16); *10, 20, 36, 37, 50, 59, 60, 66–68, 70–73, 94* (ref. 17); *12, 13, 47, 48, 51, 79–82, 84, 85, 87* (ref. 18); *42, 43, 53, 54, 78, 86* (ref. 19); *64* (ref. 20); *35, 38* (ref. 21); *6* (ref. 22); *7* (ref. 23); *58* (ref. 24); *63* (ref. 25); *89* (ref. 26); *8, 18, 44, 92* (ref. 27); *61, 62, 69, 74, 90, 91, 93* (ref. 28).

*Reference compounds.* Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide) was obtained from Calbiochem (Los Angeles, California, U.S.A.), whereas tilorone dihydrochloride [2,7-bis[2-(diethylamino)ethoxy]-fluoren-9-onedihydrochloride] was supplied by Richardson-Merrell Inc. (Cincinnati, Ohio, U.S.A.). Berenil (diminazene diaceturate) was a product of Hoechst A.G. (Frankfurt/Mainz, F.R.G.), whereas stilbamidine isethionate (4,4'-stilbenedicarboxamidine diisethionate) and pentamidine isethionate (4,'-diamidino-$\alpha,\omega$-diphenoxypentane isethionate) were products of May and Baker Ltd (Dagenham, England).

*Radiochemicals.* The radiolabelled nucleoside [*methyl*-$^3$H]dThd (specific radioactivity, 38 Ci/mmole) was obtained from the Institute of Radio Elements (IRE, Fleurus, Belgium).

*Inhibition of tumor cell growth.* All assays were performed in Linbro microplates (model FB-48-TC, Linbro Chemical Company, New Haven, Connecticut, U.S.A.). To each well were added 5 $\times$ 10$^4$ L1210 cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h at 37°C in a humidified, $CO_2$-controlled atmosphere. The growth of the cells was linear during this 48 h-incubation period. At the end of the incubation period, the cells were counted in a Coulter counter (Coulter Electronics Ltd, Harpenden Herts., England). The $ID_{50}$ (50% inhibitory dose) was defined as the concentration of compound that reduced the number of living cells by 50%.

*Inhibition of [methyl-$^3$H]dThd incorporation.* The incorporation of [*methyl*-$^3$H]dThd into cellular DNA was also measured in Linbro microplates. To each well were added 10$^5$ L1210 cells, 6.5 pmoles (0.25 $\mu$Ci) of [*methyl*-$^3$H]dThd, and a given amount of the test compound. The cells were al-

lowed to prc
humidified, C
end of this inc
wells (200 $\mu$l) v
filters (type A
Ann Arbor, M
Millipore 3025
filters were w
phate-buffered
chloroacetic ac
acetic acid, on
cold ether. The
10 min at 60°C
toluene-based s

In vivo *tests.* I
$\times$ DBA/2) mic
the *in vivo* test
and inoculated
L1210 cells per
cells were cultu
jected into mice
span (MLS) tha
ments (25 days)
if cells were us
peritoneal cavi
pounds were a
day after L121(
ceived 0.2 ml o

## Results

According to th
skeleton, the c
classes (Tables 1
ly be regarded a
in which the ami
rings are linked
carbon-oxygen)
C and D were fu
type of the hete
benzo[b]thiophe
(Table 1, 3 and 4
compounds whe
zolino-) substitu
composed of a f

lowed to proliferate for 20 h at 37°C in a humidified, $CO_2$-controlled atmosphere. At the end of this incubation period, the contents of the wells (200 µl) were brought onto 25-mm glass fiber filters (type A/E, Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.), mounted on a Millipore 3025 sampling manifold apparatus. The filters were washed twice with cold PBS (phosphate-buffered saline), twice with cold 10% trichloroacetic acid, twice with cold 5% trichloroacetic acid, once with cold ethanol, and once with cold ether. The filters were then allowed to dry for 10 min at 60°C and assayed for radioactivity in a toluene-based scintillant.

In vivo *tests*. Five- to six-week-old $BDF_1$ ($C_{57}BL$ × DBA/2) mice weighing 18 to 20 g were used for the *in vivo* tests. They were housed at 8 per cage and inoculated intraperitoneally with $5 \times 10^6$ L1210 cells per 0.2 ml PBS per mouse. The L1210 cells were cultured *in vitro* before they were injected into mice. As a consequence, the median life span (MLS) that has been observed in these experiments (25 days) is longer than it should be (7 days) if cells were used that have been passaged in the peritoneal cavity of $BDF_1$ mice. The test compounds were also injected intraperitoneally, one day after L1210 cell inoculation. Control mice received 0.2 ml of a PBS solution.

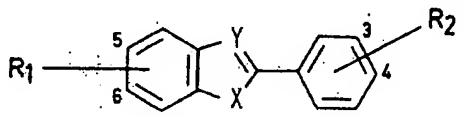
## Results

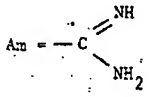
According to the type of the amidino-substituted skeleton, the compounds were divided into six classes (Tables 1 – 6). Class D (Table 4) can actually be regarded as an extension of class A (Table 1) in which the amidino- (or imidazolino-) substituted rings are linked by a carbon (carbon-nitrogen or carbon-oxygen) chain of varying length. Classes A, C and D were further subdivided according to the type of the heterocyclic ring (indole, benzofuran, benzo[b]thiophene, indene or benzimidazole) (Table 1, 3 and 4). Class B (Table 2) contains those compounds where each of the amidino- (or imidazolino-) substituted rings is a benzene. Class E is composed of a few compounds with the aryl moieties (indoles) linked together through a $C_2 - C_6$ linkage, instead of the usual $C_2 - C_2$ linkage (class C compounds, Table 3). Some miscellaneous compounds were brought together in Table 6. All compounds tested are listed in Tables 1 – 6 with their respective $ID_{50}$ (50% inhibitory dose) values for L1210 cell growth and DNA synthesis [*methyl*-$^3$H]dThd incorporation).

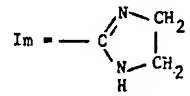
### *Inhibitory effect on L1210 cell growth*

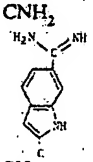
Within *class A compounds* (Table 1), the highest inhibitory activity on L1210 cell growth ($ID_{50}$: 0.21 µg/ml) was noted for compound *20*, which was about 20 times more active than the standard diarylamidine (DAPI, compound *4*). This increase in activity was probably due to substitution of amidino by imidazolino groups and not to substitution of the ring nitrogen by sulfur. Indeed, within the subclass of the benzo[b]thiophene analogues substitution of imidazolino for amidino groups resulted in a significant increase in antitumor cell activity (compare compounds *19* with *15* and *20* with *17*). Also substitution of the amidino by guanidino or $CH=NNHC(=NH)NH_2$ groups caused a marked increase in antitumor cell activity (compare compounds *12* with *9*, and *22* and *23* with *17*). For the amidino-substituted indoles, the degree of antitumor cell activity depended on the position of the amidino groups, thus in order of decreasing activity: 6,4′ > 5,4′ > 6,3′ > 5,3′ (*4* > *2* > *3* > *1*). For the amidino-substituted benzo[b]thiophenes, the position of the amidino groups did not significantly alter the antitumor cell activity (compounds *14* through *18*). Introduction of an amino group in position 3 decreased the activity (compare compounds *5* with *4*, and *18* with *15*). Molecular planarity seems to be equally important for inhibitory activity. If the planar state of compound *15* was reduced by a 1,1-dioxy-substitution (compounds *24* and *25*) and hydrogenation of carbons 2 and 3 (compound *25*), there was a concomitant reduction in inhibitory activity. In general, isosteric replacements, i.e. NH by $CH_2$ (compounds *4* and *26*) or NH by S (compounds *4* and *17*), did not significantly alter the antitumor cell activity. However, for the 5,3′ diamidines, replacement of NH by S (compounds *1* and *14*) en-

*Table 1.* Class A compounds.




| Compound no. | X | Y | $R_1$ | $R_2$ | $ID_{50}$[c] (μg/ml) | |
|---|---|---|---|---|---|---|
| | | | | | L1210 cell growth | [methyl-$^3$H]dThd incorporation |
| I. *Indoles* | | | | | | |
| *1* | NH | CH | Am[a] (5) | Am (3) | 27.0 | > 100 |
| *2* | NH | CH | Am (5) | Am (4) | 8.90 | > 100 |
| *3* | NH | CH | Am (6) | Am (3) | 15.4 | > 100 |
| *4* | NH | CH | Am (6) | Am (4) | 4.15 | > 100 |
| *5* | NH | $CNH_2$ | Am (6) | Am (4) | > 100 | > 100 |
| *6* | NH |  | Am (6) | Am (4) | > 19.4 | > 100 |
| *7* | NH | CH | Am (6) | $OCH_3$ (3) $OCH_3$ (4) $OCH_3$ (5) | 47.1 | 31.4 |
| *8* | NH | CH | Im[b] (6) | Im (4) | 2.82 | 58 |
| II. *Benzofurans* | | | | | | |
| *9* | O | CH | Am (5) | Am (4) | 7.65 | > 100 |
| *10* | O | CH | Im (5) | Im (4) | 1.52 | 14.1 |
| *11* | O | CH | Am (5) | $NH_2$ (4) | 2.19 | 26.5 |
| *12* | O | CH | $NHC(=NH)NH_2$ (5) | $NHC(=NH)NH_2$ (4) | 0.620 | 31.5 |
| *13* | O | CH | $N=CHN(CH_3)_2$ (5) | $N=CHN(CH_3)_2$ (4) | 2.30 | > 100 |
| III. *Benzo[b]thiophenes* | | | | | | |
| *14* | S | CH | Am (5) | Am (3) | 6.93 | > 100 |
| *15* | S | CH | Am (5) | Am (4) | 6.35 | > 100 |
| *16* | S | CH | Am (6) | Am (3) | 8.43 | > 100 |
| *17* | S | CH | Am (6) | Am (4) | 7.95 | > 100 |
| *18* | S | $CNH_2$ | Am (5) | Am (4) | 14.5 | > 100 |
| *19* | S | CH | Im (5) | Im (4) | 0.492 | 6.3 |
| *20* | S | CH | Im (6) | Im (4) | 0.210 | 5.0 |
| *21* | S | CH | $NHC(=NH)NH_2$ (5) | $NHC(=NH)NH_2$ (4) | 0.560 | > 100 |
| *22* | S | CH | $NHC(=NH)NH_2$ (6) | $NHC(=NH)NH_2$ (4) | 0.980 | – |
| *23* | S | CH | $CH=NNHC(=NH)NH_2$ (6) | $CH=NNHC(=NH)NH_2$ (4) | 0.590 | 27.5 |
| *24* | $SO_2$ | CH | Am (5) | Am (4) | 93.0 | > 100 |
| *25* | $SO_2$ | $CH_2$ | Am (5) | Am (4) | > 100 | > 100 |
| IV. *Indene* | | | | | | |
| *26* | $CH_2$ | CH | Am (6) | Am (4) | 6.55 | > 100 |

[a] For structure of amidino (Am), see structure over column heads.
[b] For structure of imidazolino (Im), see structure over column heads.
[c] $ID_{50}$ = inhibitory dose-50.
[d] DAPI.
All experiments were done in duplicate or triplicate.

*Table 2.* Class B c

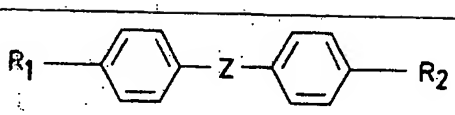
| Compound no. |
|---|
| *27*[d] |
| *28*[e] |
| *29*[f] |
| *30* |
| *31* |
| *32* |
| *33* |
| *34* |
| *35* |
| *36* |
| *37* |
| *38* |

[a-c] See corresponding
[d] *27*: stilbamidine is
[e] *28*: berenil.
[f] *29*: pentamidine is

hanced the activit

Within *class B c* cidal drug pentam displayed the great proliferation (ID trypanocidal drug isethionate (*27*) w When the bridge c contained a NH-N amidino by one or significant influen (compare compou *37*). However, wh benzenes consisted dazolino substitue tivity than did the bridges such as pyr pound *32*) or thiop inhibitory activities

*Table 2.* Class B compounds.




| Compound no. | Z | $R_1$ | $R_2$ | $ID_{50}$[c] (μg/ml) | |
|---|---|---|---|---|---|
| | | | | L1210 cell growth | [methyl-$^3$H]dThd incorporation |
| *27*[d] | CH=CH | Am[a] | Am | 22.6 | > 100 |
| *28*[e] | NH-N=N | Am | Am | 14.6 | > 100 |
| *29*[f] | $O(CH_2)_5O$ | Am | Am | 0.745 | 29.5 |
| *30* |  | Am | Am | 5.00 | 69.0 |
| *31* |  | Am | Am | 17.8 | > 100 |
| *32* |  | Am | Am | 7.70 | 91 |
| *33* |  | Am | Am | 36.7 | > 100 |
| *34* |  | Am | Am | 5.50 | > 100 |
| *35* | *p*-NHCO-$C_6H_4$-CONH | Am | Am | > 100 | > 100 |
| *36* | NH-N=N | Im[b] | Am | 17.5 | > 100 |
| *37* | NH-N=N | Im | Im | 15.8 | 66.3 |
| *38* | *p*-NHCO-$C_6H_4$-CONH | Im | Im | 3.14 | > 100 |

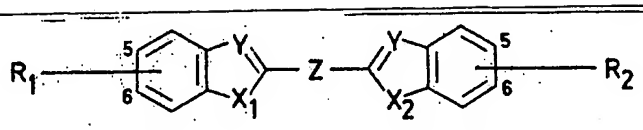
[a-c] See corresponding footnotes to Table 1.
[d] *27*: stilbamidine isethionate.
[e] *28*: berenil.
[f] *29*: pentamidine isethionate.

hanced the activity fourfold.

Within *class B compounds* (Table 2), the trypanocidal drug pentamidine isethionate (compound *29*) displayed the greatest inhibitory effect on L1210 cell proliferation ($ID_{50}$: 0.74 μg/ml). Two other trypanocidal drugs, berenil (*28*) and stilbamidine isethionate (*27*) were 20 – 30 times less inhibitory. When the bridge connecting the two benzene rings contained a NH-N=N group, substitution of the amidino by one or two imidazolino groups had no significant influence on the antitumor cell activity (compare compound *28* with compounds *36* and *37*). However, when the bridge between the two benzenes consisted of p-NHCO-$C_6H_4$CONH, imidazolino substituents conferred a much higher activity than did the amidino groups. Heterocyclic bridges such as pyrrole (compound *30*), furan (compound *32*) or thiophene (compound *34*) gave similar inhibitory activities. In contrast, an imidazole (compound *31*) or oxadiazole bridge (compound *33*) led to a decreased antitumor cell activity.

Within *class C compounds* (Table 3), three compounds (*40*, *48* and *50*) inhibited L1210 cell proliferation at an $ID_{50}$ of less than 1 μg/ml. As noted above for the class A compounds, substitution of the amidino by imidazolino groups brought about a (slight) increase in antitumor cell activity (compare compounds *50* with *41*, and *51* with *47*). The positioning of the amidino groups did not drastically influence the inhibitory effects on tumor cell growth. If the amidino groups of both benzofuran rings were moved from position 5 (compound *41*) to position 6 (compound *43*), there was a threefold decrease in inhibitory activity. However, if only one of the amidino groups was moved from position 5 (compound *41*) to position 6 (compound *42*), no substantial difference in inhibitory activity was observed. This was also the case for the positional isomers of

*Table 3.* Class C compounds.



| Compound no. | $X_1$ | Y | $X_2$ | Z | $R_1$ | $R_2$ | $ID_{50}$[c] (μg/ml) L1210 cell growth | $ID_{50}$[c] (μg/ml) [methyl-$^3$H]dThd incorporation |
|---|---|---|---|---|---|---|---|---|
| I. *Benzofuran/benzofuran* | | | | | | | | |
| *39* | O | CH | O | $CH_2$ | Am[a] (5) | Am (5) | 4.00 | > 100 |
| *40* | O | CH | O | $(CH_2)_2$ | Am (5) | Am (5) | 0.96 | 58.7 |
| *41* | O | CH | O | CH=CH | Am (5) | Am (5) | 1.51 | > 100 |
| *42* | O | CH | O | CH=CH | Am (5) | Am (6) | 1.66 | 54.8 |
| *43* | O | CH | O | CH=CH | Am (6) | Am (6) | 4.55 | 71 |
| *44* | O | $CCH_3$ | O | CH=CH | Am (5) | Am (5) | 3.09 | 48.5 |
| *45* | O | CH | O | $CH=CCH_3$ | Am (5) | Am (5) | 13.6 | > 100 |
| *46* | O | CH | O | $CH_2CHCH_3$ | Am (5) | Am (5) | 1.06 | > 100 |
| *47* | O | CH | O | $(CH=CH)_2$ | Am (5) | Am (5) | 2.54 | > 100 |
| *48* | O | CH | O | $(CH=CH)_3$ | Am (5) | Am (5) | 0.636 | 20.9 |
| *49* | O | CH | O |  | Am (5) | Am (5) | 4.78 | > 100 |
| *50* | O | CH | O | CH=CH | Im[b] (5) | Im (5) | 0.497 | 2.65 |
| *51* | O | CH | O | $(CH=CH)_2$ | Im (5) | Im (5) | 1.55 | 6.80 |
| II. *Benzofuran/indole* | | | | | | | | |
| *52* | O | CH | NH | – | Am (5) | Am (5) | 2.92 | > 100 |
| *53* | O | CH | NH | CH=CH | Am (5) | Am (5) | 1.12 | 36.0 |
| *54* | O | CH | NH | CH=CH | Am (5) | Am (6) | 1.51 | 65.2 |
| III. *Benzofuran/benzo[b]thiophene* | | | | | | | | |
| *55* | O | CH | S | CH=CH | Am (5) | Am (5) | 1.41 | > 100 |
| IV. *Benzo[b]thiophene/benzo[b]thiophene* | | | | | | | | |
| *56* | S | CH | S | CH=CH | Am (5) | Am (5) | 6.60 | > 100 |
| V. *Benzimidazole/benzimidazole* | | | | | | | | |
| *57* | NH | N | NH | CH=CH | Am (5) | Am (5) | 15.2 | 52.1 |
| *58* | NH | N | NH | NH | Am (5) | – | 18.0 | 22.4 |
| VI. *Indole/indole* | | | | | | | | |
| *59* | NH | CH | NH | $C_6H_4$-O-$C_6H_4$ | Am (6) | Am (6) | 5.31 | 72 |
| *60* | NH | CH | NH | $C_6H_4$-O-$C_6H_4$ | Im (6) | Im (6) | > 100 | > 100 |
| *61* | NH | CH | NH | – | Im (6) | Im (6) | > 100 | > 100 |
| *62* | NH | CH | NH | – | Am (6) | Am (6) | 3.53 | > 100 |
| VII. *Benzo[b]thiophene/indole* | | | | | | | | |
| *63* | S | CH | NH | CH=CH | Im (6) | Im (6) | 11.4 | 82.3 |

[a-c] See corresponding footnotes to Table 1.

indole (compounds *53* and *54*). The inhibitory activity of the bis(amidinobenzofuran) and bis(imidazolinobenzofuran) derivatives was influenced by the nature of the bridge connecting the two amidino- (or imidazolino-) substituted rings: most effective were the compounds with an ethane (compound *40*) or ethene bridge (compounds *41*, *42*, *50*, *53* – *55*). A methane bridge (compound *39*) gave a fourfold lower activity than an ethane bridge (compound *40*), whereas an isopropane bridge (compound *46*) gave about the same activity. However, a substantial decrease in activity was noted upon introduction of an isopropene bridge (compound *45*). Lengthening of the connecting ethenyl chain by one ethenyl unit did not cause marked changes in antitumor cell activity of the bis(amidinobenzofuran) derivatives

*Table 4.* Class I

Compound no.

I. *Indoles*
*64*
*65*
*66*
*67*
*68*
*69*
*70*
*71*
*72*
*73*
*74*

II. *Benzofurans*
*75*
*76*
*77*
*78*
*79*
*80*
*81*
*82*
*83*
*84*
*85*
*86*
*87*

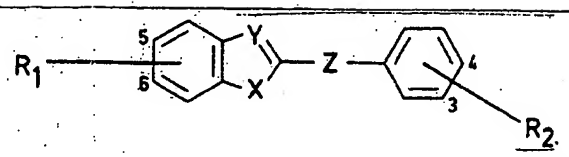
III. *Benzo[b]thio*
*88*

IV. *Benzimidazo.*
*89*

[a-c] See correspo

(compare co
although it d
dazolinobenzo
pare compou
connecting et
caused a fourf
of the bis(an

*Table 4.* Class D compounds.

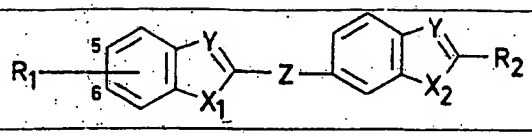



| Compound no. | X | Y | Z | $R_1$ | $R_2$ | $ID_{50}$[c] (μg/ml) | |
|---|---|---|---|---|---|---|---|
| | | | | | | L1210 cell growth | [methyl-$^3$H]dThd incorporation |
| I. *Indoles* | | | | | | | |
| *64* | NH | CH | CH=CH | Am[a] (6) | Am (4) | 3.60 | ± 100 |
| *65* | NH | CH | $C_6H_4-O$ | Am (6) | Am (4) | 1.30 | 14.8 |
| *66* | NH | CH | $C_6H_4-O$ | Am (6) | Am (3) | 0.53 | 38.6 |
| *67* | NH | CH | $C_6H_4-O$ | Am (5) | Am (4) | 1.97 | 15.3 |
| *68* | NH | CH | $CH=CH-C_6H_4-O$ | Am (6) | Am (4) | 12.4 | 62.8 |
| *69* | NH | CH | $mC_6H_4-O$ | Am (6) | Am (4) | 2.20 | 34.5 |
| *70* | NH | CH | $C_6H_4-O$ | Im[b] (6) | Im (4) | 0.50 | 13.3 |
| *71* | NH | CH | $C_6H_4-O$ | Im (6) | Im (3) | 1.49 | 21.5 |
| *72* | NH | CH | $C_6H_4-O$ | Im (5) | Im (4) | 2.17 | > 100 |
| *73* | NH | CH | $CH=CH-C_6H_4-O$ | Im (6) | Im (4) | 1.28 | 23.8 |
| *74* | NH | CH | $mC_6H_4-O$ | Im (6) | Im (4) | 3.23 | > 45.3 |
| II. *Benzofurans* | | | | | | | |
| *75* | O | CH | CH=CH | Am (5) | Am (4) | 7.30 | 60 |
| *76* | O | CH | $(CH_2)_2$ | Am (5) | Am (4) | 1.48 | > 100 |
| *77* | O | CH | $CH=CCH_3$ | Am (5) | Am (4) | 0.651 | > 100 |
| *78* | O | CH | CONH | Am (5) | Am (4) | 3.75 | > 100 |
| *79* | O | CH | CH=NNH | Am (5) | Am (4) | 3.55 | > 100 |
| *80* | O | CH | $(CH=CH)_2$ | Am (5) | Am (4) | 1.41 | 59.5 |
| *81* | O | CH | $(CH=CH)_3$ | Am (5) | Am (4) | 1.33 | 12.0 |
| *82* | O | CH | $CH=CH-C_6H_4-O$ | Am (5) | Am (4) | 1.88 | 22.0 |
| *83* | O | CH | $C_6H_4-N=NNH$ | Am (5) | Am (4) | 12.0 | 68.6 |
| *84* | O | CH | CH=CH | Im (5) | Im (4) | 0.550 | 5.55 |
| *85* | O | CH | $(CH=CH)_2$ | Im (5) | Im (4) | 1.21 | 4.30 |
| *86* | O | CH | CONH | Am (5) | Im (4) | 11.7 | > 100 |
| *87* | O | CH | $CH=CH-C_6H_4-O$ | Im (5) | Im (4) | 0.450 | 2.17 |
| III. *Benzo[b]thiophene* | | | | | | | |
| *88* | S | CH | CONH | Am (5) | Am (4) | 1.48 | > 100 |
| IV. *Benzimidazole* | | | | | | | |
| *89* | NH | N | NH | Am (5) | Am (4) | 27.8 | > 100 |

[a-c] See corresponding footnotes to Table 1.

(compare compound *41* with compound *47*), although it decreased the activity of the bis(imidazolinobenzofuran) derivative by threefold (compare compound *50* with *51*). Lengthening of the connecting ethenyl chain by two ethenyl units caused a fourfold increase in the inhibitory activity of the bis(amidinobenzofuran) derivative (compare compound *47* with *48*). In some cases, isosteric replacements caused major changes in inhibitory activity. For example, substitution of sulfur for oxygen in both benzofuran rings resulted in a four- to fivefold decrease of activity (compounds *41* and *56*). Modification of the benzofuran rings to benzimidazole rings led to a 10-fold

*Table 5.* Class E compounds.



| Compound no. | $X_1$ | Y | $X_2$ | Z | $R_1$ | $R_2$ | $ID_{50}$[c] (μg/ml) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | L1210 cell growth | [methyl-$^3$H]dThd incorporation |
| *Indole/indole* | | | | | | | | |
| *90* | NH | CH | NH | CH=CH | Im[b] (6) | Im | 24.0 | > 100 |
| *91* | NH | CH | NH | – | Im (6) | Im | 13.3 | > 100 |

[b, c] See corresponding footnotes to Table 1.

decrease of activity (compounds *41* and *57*). However, replacement of one benzofuran ring by indole (compound *53*) or benzo[b]thiopene (compound *55*) did not markedly change the antitumor cell activity. The activity of the bis(indole) derivatives was critically influenced by the nature of the substituents: in contrast to the observations made for the class A and class B compounds, substitution of imidazolino for amidino groups resulted in a complete loss of inhibitory activity (compare compounds *59* with *60*, and *61* with *62*).

*Within class D compounds* (Table 4), maximal inhibitory activity as observed for compounds *66*, *70*, *84* and *87* ($ID_{50}$: ~ 0.5 μg/ml). While substitution of both amidino by imidazolino groups caused little, if any, change in inhibitory activity for compounds *65 – 67* and *69* (compare with compounds *70 – 72* and *74*), such substitution led to a ten-fold increase in activity for compound *68* (compare with compound *73*). Among the benzofurans, the imidazolino-substituted derivatives proved, in general, more effective than the corresponding amidino-substituted derivatives (compare compounds *87* with *82*, and *84* with *75*). As demonstrated by the comparable activity of compounds *65 – 67*, on the one hand, and compounds *70 – 72*, on the other hand, the positioning of the amidino or imidazolino groups had no major impact on the cell growth-inhibiting properties of the indoles. In contrast to the class C compounds where replacement of an ethane or ethenyl by an isopropene bridge led to a considerable decrease of activity, an isopropene bridge appeared compatible with significant antitumor cell activity for class D compounds (compare compound *77* with compounds *75* and *76*). Also, lengthening of the connecting ethenyl chain by one or two ethenyl units resulted in a marked increase of activity (compare compound *75* with compounds *80* and *81*).

Of *class E* only two *compounds* (*90* and *91*) (Table 5) were tested. None showed a considerable antitumor cell activity.

Among the *miscellaneous compounds* (Table 6), the trypanocidal drug ethidium bromide (*98*) showed the highest antitumor cell activity ($ID_{50}$: 0.22 μg/ml). Compounds *97* (tilorone dihydrochloride) and *92* were also endowed with a marked inhibitory activity. Other compounds, such as the bis-amidino-substituted indoles (*93* and *94*), proved virtually inactive.

### *Inhibitory effect on L1210 cell DNA synthesis*

Of *class A compounds* (Table 1), the most potent inhibitors of L1210 cell growth, compounds *20* and *19*, showed also the strongest inhibitory effect on L1210 cell DNA synthesis. However, compound *21*, which inhibited tumor cell growth at almost the same concentration as compound *19*, did not prove effective as inhibitor of cellular DNA synthesis. In fact, most of the compounds for which the $ID_{50}$ for L1210 cell proliferation fell within the 2 – 20 μg/ml range failed to inhibit [*methyl*-$^3$H]dThd incorporation at a concentration of 100 μg/ml. Despite the

*Table 6.* Miscellar

| Compound no. | |
|---|---|
| *92* | |
| *93* | |
| *94* | |
| *95* | |
| *96* | A: |
| *97*[d] | HCl |
| *98*[e] | |

[c] See correspondin
[d] *97*: tilorone dihy
[e] *98*: ethidium bro

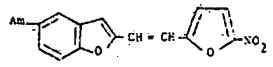
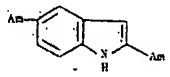
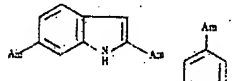
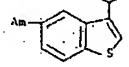
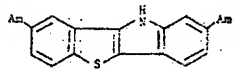
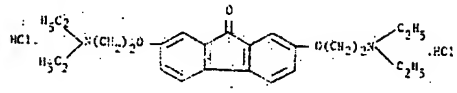
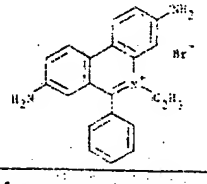
failure of several
lular DNA synth
proliferation and
corporation show
= 0.569).*

Among *class E*
potent tumor ce
isethionate (*29*),
poration only at
than L1210 cell p
pounds a corr
calculated and th

For *class C co*
tion between the

* When determining

*Table 6.* Miscellaneous compounds.

| Compound no. | Structure | $ID_{50}$[c] (μg/ml) | |
|---|---|---|---|
| | | L1210 cell growth | [methyl-$^3$H]dThd incorporation |
| *92* |  | 3.93 | 15.3 |
| *93* |  | 71.3 | > 100 |
| *94* |  | 60.5 | > 100 |
| *95* |  | 32.2 | > 100 |
| *96* |  | 8.45 | > 100 |
| *97*[d] |  | 3.15 | 14.6 |
| *98*[e] |  | 0.22 | 8.0 |

[c] See corresponding footnote to Table 1.
[d] *97*: tilorone dihydrochloride.
[e] *98*: ethidium bromide.

failure of several class A compounds to inhibit cellular DNA synthesis, their log $ID_{50}$ for L1210 cell proliferation and log $ID_{50}$ for [*methyl*-$^3$H]dThd incorporation showed a relatively close correlation (r = 0.569).*

Among *class B compounds* (Table 2), the most potent tumor cell growth inhibitor, pentamidine isethionate (*29*), inhibited [*methyl*-$^3$H]dThd incorporation only at a 40-fold higher concentration than L1210 cell proliferation. For only four compounds a correlation coefficient could be calculated and this amounted to 0.864.

For *class C compounds* (Table 3), the correlation between the log $ID_{50}$ for tumor cell growth and the log $ID_{50}$ for cellular DNA synthesis was lower than for the class A compounds (r = 0.465). Compound *50*, which was the most potent cell growth inhibitor, also exerted the strongest inhibitory effect on [*methyl*-$^3$H]dThd incorporation, although it proved about 5-fold more potent in inhibiting cell growth than DNA synthesis. Various other compounds (i.e. *41*, *46*, *47*, *55*) which inhibited tumor cell proliferation at an $ID_{50}$ comprised between 1 – 2.5 μg/ml, did not affect [*methyl*-$^3$H]dThd incorporation at a concentration of 100 μg/ml.

For *class D compounds* (Table 4), the correlation between log $ID_{50}$ for L1210 cell proliferation

* When determining the correlation coefficients, compounds with $ID_{50}$ values > 100 μg/ml were not taken into account.

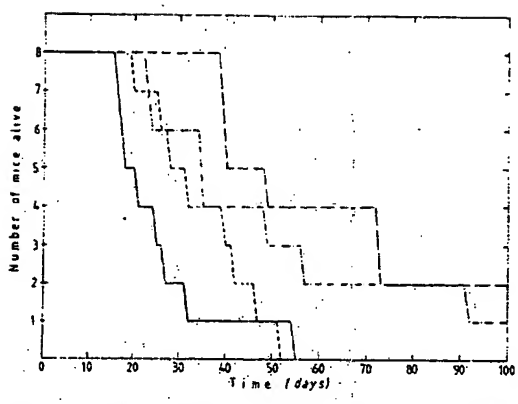



*Fig. 1.* Influence of compound *41* (diaceturate salt) on the survival of $BDF_1$ mice inoculated intraperitoneally with $5 \times 10^6$ L1210 cells per mouse. Compound *41* was injected intraperitoneally as a single dose of either 200 mg/kg (— —), 40 mg/kg (–.–.–), 8 mg/kg (– – – –) or 0 mg/kg (control) (——), one day after tumor cell inoculation.

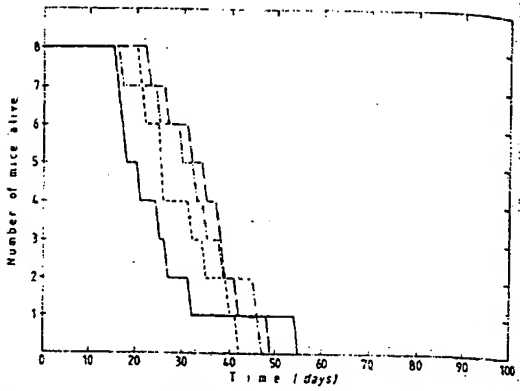



*Fig. 2.* Influence of ethidium bromide (*98*) on the survival of $BDF_1$ mice inoculated intraperitoneally with $5 \times 10^6$ L1210 cells per mouse. Ethidium bromide was injected intraperitoneally as a single dose of either 20 mg/kg (— —), 4 mg/kg (–.–.–), 0.8 mg/kg (– – – –) or 0 mg/kg (control) (——), one day after tumor cell inoculation.

and log $ID_{50}$ for [*methyl*-$^3$H]dThd incorporation was higher than for the class A and C compounds ($r = 0.706$).

Among *class E and the miscellaneous compounds* (Tables 5 & 6), ethidium bromide (compound *98*) turned out to be the most potent inhibitor of L1210 cell DNA synthesis. However, its $ID_{50}$ value for [*methyl*-$^3$H]dThd incorporation was still 40 times higher than its $ID_{50}$ value for L1210 cell proliferation, and differed not much from the $ID_{50}$ values for compound *97* (tilorone dihydrochloride) and compound *92*, two compounds which were about 10 times less potent as L1210 cell growth inhibitors than ethidium bromide. Taking together all compounds belonging to either class A, B, C, D or E, we found a correlation coefficient of 0.612.

*Antitumor activity* in vivo

Since compound *41* ranked among those diarylamidine derivatives that showed potent antitumor cell activity *in vitro* ($ID_{50}$: 1.51 μg/ml), it was further evaluated for its antitumor activity *in vivo*. $BDF_1$ mice were injected intraperitoneally with $5 \times 10^6$ L1210 cells per mouse. The median life span of these mice was 25.0 days. Compound *41* was injected intraperitoneally as a single dose of 200 mg/kg, 40 mg/kg or 8 mg/kg, one day after tumor cell inoculation. As reference compound served ethidium bromide (*98*) which was administered at 20 mg/kg, 4 mg/kg or 0.8 mg/kg. Because of toxicity higher doses of this compound could not be administered.

The antitumor effects obtained with compounds *41* and *98* are presented in Figs. 1 and 2. Compound *41* proved quite efficient in prolonging the life span of tumor cell-inoculated mice. At a dose of 200 mg/kg it increased the median survival time up to 51 days (or 204% of control) and 2 out of 8 mice survived even at 100 days after tumor cell inoculation. At a 5-fold lower dose, compound *41* achieved a median survival time of 175%, and at 8 mg/kg, it increased the life span to 139%. Ethidium bromide (*98*) proved inferior to compound *41* in increasing the life span of L1210 cell-inoculated $BDF_1$ mice. When injected at the highest tolerated dose (20 mg/kg), compound *98* demonstrated an increase of the median survival time to 137%. At lower doses (4 mg/kg and 0.8 mg/kg) compound *98* extended the median life span to 132% and 118%, respectively.

## Discussion

For a series o
zoline) deriva
according to
strongest inhi
proliferation
2-yl)-2-[4-(2-ir
phene (*20*) am
midine isethio
with 5,5'-bis
1-benzofuran
5-(2-imidazol
phenoxy]-styry
compounds ar
imidazolin)-2-
E compounds.
of these compo
responded to
pound, ethidiu
tween 0.2 and
Although th
growth were
values for [*me*
titumor cell a
diarylimidazol
cribed to their
synthesis. Inde
pounds for L1
[*methyl*-$^3$H]dT
reasonable cor
pounds togethe
the diarylamidi
pairment of DN
result from bin
pairs in DNA.
Previous stu
tiproteolytic an
oncornaviral D
tifungal and
diarylamidine d
*al.* (10) indicate
diarylamidines
ticoagulant act
tiprotease activi
depending on
(trypsin, throm

## Discussion

For a series of 96 diarylamidine (and diarylimidazoline) derivatives, divided into five classes (A – E) according to the nature of the aromatic rings, the strongest inhibitory effects on murine L1210 cell proliferation were observed with 6-(2-imidazolin-2-yl)-2-[4-(2-imidazolin-2-yl)phenyl] benzo[b]thiophene (*20*) among class A compounds, with pentamidine isethionate (*29*) among class B compounds, with 5,5'-bis-(2-imidazolin-2-yl)-2,2'-vinylenedi-1-benzofuran (*50*) among class C compounds, with 5-(2-imidazolin-2-yl)-1-(4-[4-(2-imidazolin-2-yl) phenoxy]-styryl)-1-benzofuran (*87*) among class D compounds and with 2-(2-imidazolin-2-yl)-6-[6-(2-imidazolin)-2-yl)-2-indolyl]indole (*91*) among class E compounds. The cell growth-inhibiting potency of these compounds (with the exception of *91*) corresponded to that of the known cytotoxic compound, ethidium bromide (*98*) ($ID_{50}$ ranging between 0.2 and 0.75 mg/ml).

Although the doses required to inhibit L1210 cell growth were considerably lower than the $ID_{50}$ values for [*methyl*-$^3$H]dThd incorporation, the antitumor cell activity of the diarylamidines and diarylimidazolines may at least partially be ascribed to their suppressive effects on cellular DNA synthesis. Indeed, the log $ID_{50}$ of the test compounds for L1210 cell growth and the log $ID_{50}$ for [*methyl*-$^3$H]dThd incorporation showed a reasonable correlation: $r = 0.612$ for all compounds together. Thus, the cytotoxic properties of the diarylamidine derivatives may be due to an impairment of DNA replication, which, in turn, may result from binding of the diamidines to A – T base pairs in DNA.

Previous studies have been focussed on the antiproteolytic and anticoagulant activities (10), anti-oncornaviral DNA polymerase activity (9) and antifungal and antibacterial activities (29) of diarylamidine derivatives. The studies of Tidwell *et al.* (10) indicated that the antiproteolytic activity of diarylamidines did not correlate with their anticoagulant activity and that even for the antiprotease activity marked differences were noted, depending on the nature of the protease tested (trypsin, thrombin or kallikrein). A comparison of structure-activity data with those previously reported by Tidwell *et al.* reveals that there is no direct correlation between anti-protease activity and anti-L1210 cell proliferation activity. For example, compound *10* was inactive as a trypsin inhibitor, yet fairly active as an L1210 cell growth-inhibitor. Whereas substitution of imidazolino for amidino generally increased the L1210 cell-growth inhibiting activity (e.g. compare compound *9* with *10*, *15* with *19*, *17* with *20*, etc.), it annihilated the anti-protease activity (compare compound *14* with *17*, and compound *24* with *32* in ref. 10). Furthermore, some very effective trypsin inhibitors (compounds *10* and *29* in ref. 10) were inactive as inhibitors of L1210 cell proliferation (compounds *5* and *25* in Table 1).

Similarly, no direct correlation could be established between the inhibitory effects of the diarylamidine derivatives on oncornaviral DNA polymerase (9) and their L1210 cell-inhibiting properties. For example, compound *27* in ref. 9, which proved barely active against oncornaviral DNA polymerase, exhibited the greatest L1210 cell-growth inhibiting activity among the class B compounds (compound *29* in Table 2). Also, compound *21*, one of the most potent inhibitors of L1210 cell proliferation, was only weakly active as inhibitor of the oncornaviral DNA polymerase activity (compound *19* in ref. 9). Compound *83*, an efficient inhibitor of oncornaviral DNA polymerase (compound *69* in ref. 9), showed only moderate activity as an inhibitor of L1210 cell proliferation.

While Anné *et al.* (29) observed similarities in the structural requirements for the antibacterial and antifungal properties of the diarylamidine derivatives, these structural similarities do not extend to the antitumor cell activity of the diarylamidines. For example, substitution of amidino by imidazolino groups diminished the antifungal activity (compare compounds *60* with *56*, *59* with *57*, *71* with *62*, and *72* with *67* in ref. 29), but enhanced the L1210 cell growth-inhibiting activity (compare compounds *19* with *15*, *20* with *17*, *38* with *35*, *70* with *65*, and *84* with *75*). Compounds *4* and *27*, which proved to be the most potent antifungal compounds among class A and class B compounds

(compound *4* and *25* in ref. 29), respectively, were only weakly active as inhibitors of L1210 cell proliferation.

Thus, the structure-function relationship data obtained in this area and previous studies (9, 10, 29) point to important differences in the structural requirements of diarylamidine derivatives as either protease inhibitors or DNA polymerase inhibitors, or antifungal, antibacterial, or antitumor agents. The antitumor activity that has been demonstrated with compound *41 in vivo* ($BDF_1$ mice inoculated with L1210 cells) suggested that further antitumor studies should be undertaken with those compounds that show the greatest potency as inhibitors of L1210 cell growth *in vitro*. These antitumor studies should also be extended to animal models other than the L1210 system.

## Acknowledgements

We are indebted to Lizette van Berckelaer for excellent technical assistance and to Christiane Callebaut for skilled editorial help.

This study was supported by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Krediet no. 30048.75) and Geconcerteerde Onderzoeksacties (Conventie no. 76/81-IV), and from the Wilhelm-Sander-Stiftung.

## References

1. Newton BA: Chemotherapeutic compounds affecting DNA structure and function. Adv Pharmacol Chemother 8:149–184, 1970
2. Kapuscinski J, Szer W: Interactions of 4',6-diamidine-2-phenylindole with synthetic polynucleotides. Nucleic Acids Res 6:3519–3534, 1979
3. Kapuscinski J, Yanagi K: Selective staining by 4',6-diamidine-2-phenylindole of nanogram quantities of DNA in the presence of RNA on gels. Nucleic Acids Res 6:3535–3542, 1979
4. Grossgebauer K, Kegel M, Dann O: Neues fluoreszenzoptisches Verfahren in der medizinisch-diagnostischen Mikrobiologie. Deutsche Medizinische Wochenschrift 29:1098–1099, 1976
5. Schnedl W, Mikelsaar A-V, Breitenbach M, Dann O: DIPI and DAPI: fluorescence banding with only negligible fading. Hum Genet 36:167–172, 1977
6. Chandra P, Mildner B, Dann O, Metz A: Influence of 4',6-diamidino-2-phenylindole on the secondary structure and template activities of DNA and polydeoxynucleotides. Mol Cell biochem 18:81–86, 1977
7. Mildner B, Metz A, Chandra P: Interaction of 4',6-diamidino-2-phenylindole to nucleic acids, and its implication to their template activity in RNA-polymerase reaction of E.coli bacteria of Friend-virus infected mouse spleen. Cancer Letters 4:89–98, 1978
8. Müller W: Über die Bindung trypanociden Diamidine an Desoxirobonucleinsäuren (DNA). Proc Symp "Trypanocide, fluoreszierende Diamidine", Erlangen (W.-Germany), 19–20 February 1982
9. De Clercq E, Dann O: Diarylamidine derivatives as oncornaviral DNA polymerase inhibitors. J Med Chem 23:787–795, 1980
10. Tidwell RR, Geratz JD, Dann O, Volz G, Zeh D, Loewe H: Diarylamidine derivatives with one or both of the aryl moieties consisting of an indole or indole-like ring. Inhibitors of arginine-specific esteroproteases. J Med Chem 21:613–623, 1978
11. Dann O, Bergen G, Demant E, Volz G: Trypanocidal diamidines of 2-phenyl-benzofurans, 2-phenyl-indenes and 2-phenyl-indols. Justus Liebigs Ann Chem 68–89, 1971
12. Dann O, Fernbach R, Pfeifer W, Demant E, Bergen G, Lang S, Lürding G: Trypanocidal diamidines with three rings in two isolated ring systems. Justus Liebigs Ann Chem 37–87, 1972
13. Dann O, Hieke E, Hahn H, Miserre HH, Lürding G, Rössler R: Polynuclear thiophenes. VIII. Trypanocidal diamidines of 2-phenylthionaphthene. Justus Liebigs Ann Chem 23–45, 1970
14. Dann O, Fick H, Pietzner B, Walkenhorst E, Fernbach R Zeh D: Trypanocidal diamidines with three isolated ring systems. Justus Liebigs Ann Chem 160–194, 1975
15. Dann O, Volz G, Demant E, Pfeifer W, Bergen G, Fick H, Walkenhorst E: Trypanocidal diamidines with four rings in one or two ring systems. Justus Liebigs Ann Chem 1112–1140, 1973
16. Dann O, Char H, Griessmeier H: Synthesis of biscationic trypanocidal 1-benzofuran-compounds. Justus Liebigs Ann Chem 1836–1869, 1982
17. Ruff J: Ph.D. Thesis, University of Erlangen-Nürnberg, 1979
18. Griessmeier H: Ph.D. Thesis, University of Erlangen-Nürnberg, 1979
19. Char H: Ph.D. Thesis, University of Erlangen-Nürnberg, 1978
20. Wolff HP: Ph.D. Thesis, University of Erlangen-Nürnberg, 1977
21. Wander A: British Patent 1007–334. Chem Abstr 64:5102, 1966 [Compound 35 was prepared anew by J. Ruff, whereas compound 38 was a gift from Dr. R. Fischer (Wander, A.G., Bern)]
22. Stich W: Ph.D. 1981
23. Holzheid R: Nürnberg, 198
24. Schünger G: Nürnberg, 198
25. Fleischmann P Nürnberg, 1981

22. Stich W: Ph.D. Thesis, University of Erlangen-Nürnberg, 1981
23. Holzheid R: Ph.D. Thesis, University of Erlangen-Nürnberg, 1982
24. Schünger G: Ph.D. Thesis, University of Erlangen-Nürnberg, 1982
25. Fleischmann-P: Ph.D. Thesis, University of Erlangen-Nürnberg, 1981
26. Walkenhorst E: Ph.D. Thesis, University of Erlangen-Nürnberg, 1970
27. Gift from Dr. G. Volz
28. Gift from Dr. J. Rüff
29. Anné J, De Clercq E, Eyssen H, Dann O: Antifungal and antibacterial activities of diarylamidine derivatives. Antimicrob Agents Chemother 18:231–239, 1980

**This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record**

## BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☑ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☑ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

**IMAGES ARE BEST AVAILABLE COPY.**
**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**